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PRINCIPAL INVESTIGATOR: Quingshen Gao, M.D.

CONTRACTING ORGANIZATION: New England Medical Center  
Hospitals, Incorporated  
Boston, Massachusetts 02111

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<b>13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)</b> In the approved Statement of Work, we proposed three tasks for the first years. We have successfully accomplished Task 1 and 2. We already successfully constructed the positive-negative selection construct that was proposed in our grant application. We also constructed the positive control construct and standardized the PCR screen conditions. We confirmed the status of BRCA2 in a normal mammary epithelial cell strain, 76N is normal and is suitable to be used to generate the BRCA2 +/- cell line. However, Task 3 turned out to be much more difficult than we have anticipated. Thus far we have not yet obtained clones with one allele of BRCA2 disrupted even after numerous attempts. We are currently in the process of more attempts with or without modified strategy. We already generated two promoterless constructs with puromycin and neomycin resistance gene respectively, which has been reported to have a higher success rate. We already transfected both constructs into 76TERT cells and are in the process of selection. If we are able to obtain the BRCA2 +/- cells, we will perform the radiation transformation experiments as we proposed. Otherwise, we will construct the set of knock out construct to target the exon 3 region of BRCA2.				
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## INTRODUCTION

Breast cancer is by far the most frequently diagnosed cancer in women. Each year over 186,000 new cases and 46,000 deaths are reported in United State alone(1). Germ-line mutations in the breast cancer susceptibility genes BRCA2 and BRCA1 confer susceptibility to familial early-onset breast and ovarian cancers (2-6). So far very few somatic mutations of BRCA2 have been found in sporadic breast cancer. However, loss of heterozygosity at the BRCA2 locus has been observed in 30-40% of sporadic breast tumors (7-12), which suggests that loss of BRCA2 gene may also be associated with sporadic breast and ovarian cancers. It has been noted that the genomic region of BRCA1 and BRCA2 genes contains very high densities of repetitive DNA elements, which can lead to high genomic instability (13). This distinctive genomic instability might contribute to the inactivation of BRCA2 in sporadic breast or ovarian cancer (13).

Extensive studies indicated that BRCA2 plays an important role in DNA repair. Several studies found that BRCA2 binds to BRCA1 and Rad51 and co-localizes with them in the nucleus (14-17). Rad51 is a homologue of the *Escherichia coli recA* gene. Rad51 is known to function in recombinational repair of double strand DNA breaks (18-21). Binding of BRCA2 to Rad51 suggests that BRCA2 and Rad51 might function in the same pathway. Studies with BRCA2 knock out mice further confirmed the role of BRCA2 in DNA repair. Sharan et al first reported that BRCA2 null mouse embryos are hypersensitive to  $\gamma$ -radiation (14). These data were further confirmed with embryonic stem (ES) cells and mouse embryonic fibroblasts (MEF) with less severe BRCA2 truncations (22-24). Particularly, spontaneous accumulation of chromosomal abnormalities, including breaks and aberrant chromatid exchanges was observed in BRCA2 null MEFs(23). The human pancreatic tumor cell line, Capan-1, was found to have the 6174delT mutation in its BRCA2 gene and loss of the other allele (25-27). In correlation with the observation in BRCA2 knock-out MEFs, Capan-1 cells are extremely sensitive to ionizing radiation and DNA damaging agents (26-27). Importantly, reconstituted expression of wild-type BRCA2 in Capan-1 cells confers to these cells resistance to DNA damaging agents (27). Furthermore, antisense BRCA2 deoxyribonucleotides that can decrease the expression of BRCA2 RNA in cells significantly increase the sensitivity of these cells to DNA damaging agents (26). Additionally, overexpression of a BRC repeat in MCF-7 cells (BRCA2<sup>+/+</sup>) that attenuates the function of BRCA2 rendered these cells sensitive to DNA damaging agents, and abrogated the G2-M checkpoint induced by DNA damage (28). Recent studies directly demonstrated that BRCA2 plays a role the homology-directed recombinational repair of double strand DNA breaks (29-32). Furthermore, two recent studies provide further structural confirmation for this role of BRCA2 (33-35). These data clearly underscores the roles of BRCA2 in DNA damage response in human cells.

In addition to surgery and systemic therapy, radiation therapy is one of the most important components of breast cancer management. Lumpectomy and radiation therapy (LRT) remains a standard of care option for the majority of patients with early stage breast cancer. It is estimated that about 40-60% breast cancer patients with early stage disease will undergo radiation treatment (36-38). However, development of radiation-

induced secondary malignancy after successful radiotherapy of a prior cancer has been well documented. It is generally accepted among radiation oncologists that for most patients this is an unavoidable complication (39). However, for a specific population of patients, such as BRCA2 or BRCA1 germline mutation carriers, the risk of radiation induced breast cancer warrants further investigation.

Since BRCA2 has been proven to play a important role in the double stranded DNA damage repair, breast cancer patients with a germline BRCA2 mutation may be more susceptible to radiation induced transformation. First, this could be due to haploinsufficiency. Although the classic Knudson's two-hit model of tumorigenesis stipulates that mutation of both alleles of a tumor suppressor gene is needed to trigger tumor formation, a number of studies indicated that mutation or loss of a single allele may be sufficient to exert a cellular phenotype that leads to tumorigenesis without inactivation of the second allele. Recently, haploinsufficiency of BLM gene, another tumor suppressor gene product that is involved in DNA repair, have been demonstrated to enhance tumorigenesis. It has been a puzzling observation that while loss of heterozygosity at the BRCA2 locus has been detected in 30-40% of sporadic breast and ovarian tumors, very few mutations were ever found in these sporadic cases (8-11) (40). These studies imply that deletion of one copy of BRCA2 can also compromise some functions of BRCA2 and permit tumorigenesis. Notably, in mouse knock out system, there is no statistically significant difference observed between BRCA2 wild type and BRCA2 heterozygous embryos or cells in their sensitivity to DNA damaging agents (14, 22-24). However, the response of human mammary epithelial cells could be very different from mouse ES cells or MEFs, and should be directly assessed. Furthermore, it should be noted that the sensitivity to radiation is different from the susceptibility to radiation-induced transformation. A slight increase in radiation sensitivity, which may escape from detection, could lead to more mutations to be accumulated than a more significant increase in radiation sensitivity, which usually eliminated the mutated cells. Interestingly, Patel et al consistently observed a small difference between wild-type and heterozygous cells in their sensitivity to DNA damaging agents (23). Secondly, even without haploinsufficiency, because the BRCA2 gene occupies a large locus, cells with only one copy of BRCA2 will be more vulnerable to inactivation by radiation. Once the only wild type copy of BRCA2 gene is inactivated by radiation, the DNA repair will be impaired, further mutations will accumulate, leading to transformation.

To test this hypothesis that radiation treatment of breast cancer patients who inherit one defective copy of the BRCA2 gene may pose greater risk of radiation-induced secondary cancer, we propose to generate BRCA2<sup>+/-</sup> cells from normal human mammary epithelial cells, and directly compare the susceptibility of the BRCA2<sup>+/-</sup> cells and their parental cells to radiation induced transformation.

Clinical study would have been a good system to test this hypothesis, however, it is hindered by small numbers of patients with documented BRCA2 status, long term follow up, and interference of multigenic effects.

Knock out mouse systems has very limit value. The studies that are carried out in mouse knock out system, especially in fibroblasts of mouse origin may not be correct in human mammary epithelial system. Mouse cells are very susceptible to transformation by chemical carcinogens, radiation, tumor viruses or oncogenes. Spontaneous immortalization and transformation can be obtained easily. In contrast, human cells are very resistant to transformation by all the transforming agents. Spontaneous immortalization almost never occurs in human cell culture system (41-42). Also, BRCA2 is poorly conserved from mouse to human. Comparison of the amino acids sequence of human BRCA2 and mouse BRCA2 indicated that human BRCA2 exhibits only 59% identity with mouse BRCA2, while the other tumor suppressor genes, such as APC, WT1, NF1, and RB1 exhibit greater than 90% identity (43).

One pair of normal human mammary epithelial cell strains with identical genetic background and different BRCA2 status will be an ideal system to assess the susceptibility of BRCA2 heterozygous normal human mammary epithelial cells to radiation induced transformation. The susceptibility to radiation-induced transformation can be affected by many genetic factors. Knocking out one copy of BRCA2 from a mammary epithelial cell strain with normal BRCA2 genotype will generate a normal mammary epithelial cell strain with identical genetic background to its parental strain with the exception of being BRCA2 heterozygous. These BRCA2<sup>+/-</sup> cells and their parental cells will be an ideal system to study the effects of being BRCA2 heterozygous on the susceptibility to radiation-induced transformation. As the parental cells are very resistant to radiation induced transformation (unpublished observation), the effects of being BRCA2 heterozygous will be easily assessed. In this application we proposed to generate a BRCA2 heterozygous mammary epithelial cell strain, and directly compare the susceptibility of the BRCA2<sup>+/-</sup> cells and its parental cells to radiation-induced transformation (see Fig 1).

Somatic cells knock out technology is the most direct and unambiguous way to eliminate the function of a gene. In spite of many recent advances, somatic cells knock out remains a very difficult and arduous approach, which is still considered complex and high-risk. The reason for this is that in mammalian cells nonhomologous recombination is orders of magnitude more frequent than homologous recombination. Two approaches have been developed to enrich the homologous recombination (44, 45). The first approach is the "positive-negative" selection method. The negative selection can prevent non-homologous recombinants from scoring as colonies. The second approach is the so called promoterless approach, in which the expression of resistance gene is conditional on recombination at the homologous target site. We proposed the "positive-negative" method in our original proposal because more successful reports were published. We also planned the promoterless method, which is reported to have a better success rate.

In the approved Statement of Work, we proposed three tasks for the first years:

Task 1. Generate the BRCA2 Knock out construct and the positive control template constructs, sequence the construct. (months1-6)

Task 2. Confirm the status of BRCA2 is normal in normal human mammary epithelial cells. (months1-3)

Task 3. Transfect the knock out construct into normal mammary epithelial cells and screen for clones with one allele of BRCA2 disrupted. (months7-12)

We have successfully accomplished Task 1-2. We already successfully constructed the positive-negative selection constructs that we proposed in our grant application. We also constructed the positive control construct. Both constructs have been sequenced and proven to be correct constructs. We also standardized the PCR condition to screen for the positive clones. We confirmed the status of BRCA2 in a normal mammary epithelial cell strain, 76N is normal, and is suitable to be used to generate the BRCA2 <sup>+/-</sup> cell line. Task 3 turned out to be much more difficult than we have anticipated. Thus far we have not yet obtained clones with one allele of BRCA2 disrupted after numerous attempts. We are currently in the process of more attempts with or without modified strategy.

## REPORT BODY

### **Task 1: Generate the BRCA2 Knock out construct and the positive control template construct, and sequence the constructs. (months1-6).**

**A. Generation of the knockout construct.** We already successfully accomplished this task. As depicted in Fig. 2, a ~ 2.7 kb fragment (restriction site SacII and BamHI added) was generated by PCR from part of exon 11 and intron 11 using genomic DNA from normal mammary epithelial cells, 76N as templates ( primer 3koup and 3kodn1 was used). The resulting PCR fragment was cloned into SacII and BamHI digested pKO-NTKV (see Fig. 2b) as the short arm. A ~5 kb fragment (restriction site BglII and XhoI added) was generated by PCR from part of intron 10 and exon 11 with primer 5koup and 5kodn. The resulting fragment was cloned into pKO-NTKV as the long arm. A stop codon was added just before the XhoI site. The Neomycin gene in this vector is under the control of PGK promoter and with the polyadenylation signal of the bovine growth hormone. The transcription direction will be opposite to the transcription of BRCA2. As shown in Fig. 2c, the final knockout construct pKO-NTKV-BRCA2 exhibits the expected restriction fragments. In lane 1, pKO-NTKV-BRCA2 was digested with BamHI and XhoI, a expected 1.6 kb fragment containing the neomycin resistance gene was obtained. In lane 2, digestion by XhoI plus BglII released the 2.75 kb short arm. In lane 3, digestion by SacII plus BamHI released the 5.28 kb long arm. In lane 4, pKO-NTKV-BRCA2 was digested by all four enzymes, XhoI, BglII, SacII and BamHI, release the 2.75 kb short arm, the 5.28 kb long arm and the neomycin fragment. Furthermore, the gene boundaries of the final knock out construct pKO-NTKV-BRCA2 were fully sequenced, and expected sequence was obtained.

**B. Generation of the positive control construct.** A positive control construct was also successfully generated. As illustrated in Fig. 2a, ~3.1 kb fragment (restriction site SacII and BamHI added) was generated from part of exon 11 and intron 11 with primer 3koup and 3kodn3. The resulting PCR fragment was cloned into SacII and BamHI digested pKO-NTKV to generate the positive control construct pKO-NYKV-BRCA2-3ko3. Both ends of the insert in pKO-NYKV-BRCA2-3ko3 were sequenced and the expected sequence obtained.

**C. Standardization of the PCR screen condition.** Primer Neo-Ns and 3kodn2 were synthesized to be used to screen for the correctly recombined clones (Fig. 2a). Various amount of the positive control plasmid (from 9 copies to 9000 copies) was mixed with genomic DNA from 76N to be used to optimize the PCR condition. Our positive control plasmid, pKO-NYKV-BRCA2-3ko3 is about 15kb. Therefore,  $1.5 \times 10^{-7}$   $\mu$ g will be about 9000 copies of plasmid. We mixed  $1.5 \times 10^{-7}$   $\mu$ g,  $1.5 \times 10^{-8}$   $\mu$ g,  $1.5 \times 10^{-9}$  or  $1.5 \times 10^{-10}$   $\mu$ g of pKO-NYKV-BRCA2-3ko3 plasmid with 2  $\mu$ g of genomic DNA to be used as the positive control template to standardize the PCR conditions, which turned out to be more difficult than expected. We tried five different kinds of polymerases (Amplitag polymerase from PerkinElmer, Advantage cDNA polymerase from Clontech, Taq polymerase from Sigma and Expand Long Template PCR System from Roche) under



many PCR conditions. We found that Expand Long Template PCR System works the best. We are able to detect 9 copies of positive plasmid mixed in 2 µg of genomic DNA using the following condition (Fig. 3.): initial denaturation 94°C 2 min, denaturation 94°C 20 seconds, Annealing 65°C 20 seconds, elongation 68°C 2 minutes, 10 cycles, then denaturation 94°C 20 seconds, Annealing 65°C 20 seconds, elongation 68°C 2 minutes + 20 s for each successive cycle, 30 cycles.

**Task 2: Characterization of BRCA2 gene in a normal human mammary epithelial cell strain.** A normal mammary epithelial cell strain, 76N cells were obtained from Dr. Vimla Band. To ensure the BRCA2 gene in this normal mammary epithelial cell strain is normal, the genomic DNA from 76N cells was submitted to Myraid Genetic Laboratories, Inc, and the exons and the intron-exon junctions BRCA2 gene was fully sequenced. Two specific genetic variants were found: I2944F and I3412V. Both of these mutations are not known to be deleterious. Therefore this strain is suitable to be used to generate the BRCA2<sup>+/-</sup> cell line.

**Task 3. Transfect the knock out construct into normal mammary epithelial cells and screen for clones with one allele of BRCA2 disrupted.**

**A. Transfect the knock out construct into normal mammary epithelial cells and screen for clones with one allele of BRCA2 disrupted.** The knock out construct, pKO-NYKV-BRCA2 was linearized by NotI digestion and transfected into normal mammary epithelial cells, 76N cells with Fugene. We have a standardized protocol to transfect the normal mammary epithelial cells with Fugene. We are able to achieve a transfection efficiency of more than 50%. Forty-eight hours after transfection, cells were selected in G418 at 100 µg/ml. About 800 colonies were selected out. The colonies were pool together to test if correctly recombinant colonies are present in the pooled colonies. Although we are able to detected 9 copies of positive plasmid mixed with 2 µg genomic DNA, we did not detect the correctly recombinant colonies present in the pooled population. This experiment was repeated, however, still no positive colonies were detected.

**B. Spontaneous immortalization of 76N cells transfected by the BRCA2 knockout constructs.** Although we can not detect the positive recombined colonies, theoretically, there exist some correctly recombined colonies. If knockout of one allele of BRCA2 render the cells susceptible to spontaneous immortalization of normal mammary epithelial cells, long term culture of the 76N cells that were transfected with the BRCA2 knockout construct should select out the spontaneous immortalized cells. For this purpose, we cultured the pool population of the transfected cell for 7 month (about 9 passages), however, no spontaneous immortalization was observed.

**C. Transfect the knock out construct into hTERT immortalized normal mammary epithelial cells and screen for clones with one allele of BRCA2 disrupted.** Since we did not obtained correctly recombined BRCA2 knock out cells with normal mammary epithelial cells, 76N cells. We want to exclude the possibility of proliferation potential being the cause of failure. We attempted the knock out experiments in hTERT

immortalized cells, 76TERT cells. 76TERT cells were derived from normal human mammary epithelial cells, 76N cells. We transfected the linearized pKO-NYKV-BRCA2 construct into 76TERT cells. The transfected cells were selected in G418. The selected cells were pooled and the positive colonies were screened by PCR using the standardized condition. We attempted this experiment three times. We did not obtain any positive colony. These failures prompt us to improve our knock out construct as described in the following.

**D. Generation of promoterless knock out constructs.** Although the positive-negative selection knock out constructs has been used successfully in many cases, it was reported that the promoterless knock out construct configuration provide much better chances of success (44). To construct the promoterless knock out construct, we cloned the selection marker genes of Neomycin and Puromycin from pIRESneo and pIRESpuro into pKO-NYKV-BRCA2 to replace the original Neo expression cassette ( Fig. 4a). The pIRESpuro or pIRESneo vectors contain the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV), which permits the translation of two open reading frames from one messenger RNA. The ECMV IRES followed by the gene encoding puromycin resistance (puromycin-N-acetyl-transferase) or neomycin resistance, and the polyadenylation signal of the bovine growth hormone. Correct homologous recombination will result in a modified BRCA2 gene that can transcribe a bicistronic mRNA. Ribosomes can enter the bicistronic mRNA either at the 5' end to translate the gene of BRCA2 or at the ECMV IRES to translate the antibiotic resistance marker. Since the selection gene lacks its own promoter, the expression of the resistance marker genes is only activated from the BRCA2 promoter following correct homologous integration. We are currently in the process of selection to isolate resistant colonies. Similarly, the corresponding positive control plasmids will be also constructed as shown in Fig. 4b, which is still a work in progress.

Since we did not detect any positive recombination in many experiments. The locus we selected could be the culprits. It has been reported that the frequency of homologous recombination can be influenced by the position of targeting locus. Therefore, we have chosen another region of BRCA2 gene to design another set of knock out construct. We already have the primers designed and synthesized. As shown in Fig. 5, a short 5' arm will be generated from part of intron 2 and exon 3 by PCR with ClaI and XhoI restriction site incorporated. A stop codon was added to stop the translation of BRCA2 just before XhoI restriction site. A long 3' arm will be generated from BRCA2 gene covering part of exon 3, intron 3, exon 4, intron 4, exon 5, intron 5, exon 6 and part intron 7 (BglII and BamHI restriction site will be added). The resulting fragment will be cloned into pKO-NYKV sequentially. One set of positive control plasmids will be also constructed by generating a longer 5' arm by PCR as illustrated in Fig. 5. This set of construct will be our backup constructs in case we still do not obtain positive clones using the promoterless construct described above.

**D. The RNAi approach.** It is reported that synthetic short 21- to 22 nucleotide interfering RNAs (siRNAs) can mediate strong and specific suppression of gene expression in mammalian cells (46). This method represents a powerful tool to knock out

the function of a gene, although it can only reduce the gene expression transiently (46). Recently, a mammalian expression vector system that directs the synthesis of siRNA-transcripts (pSUPER) was reported (47, 48). Stable expression of siRNAs using this vector mediates persistent suppression of gene expression (47, 48). To establish an alternative approach to knock out the expression of BRCA2, we designed two RNAi oligonucleotides targeting two positions on the BRCA2 gene (nt. 444 to 463 and nt. 1335 to 1354). A control oligonucleotide was also designed. These oligonucleotides were annealed and cloned into pSUPER. The resulting plasmids were sequenced to ensure that they lack PCR generated mutations. We transfected these two plasmids and the control vector into 76TERT cells to assess the effect of these two RNAis. Cells were harvested 72 hours after transfection and subjected to Western Blotting analysis by anti-BRCA2 antibody Ab1 (Oncogen science). As shown in Fig. 5, BRCA2-444 can reduce the expression of BRCA2 by 65% comparing to the vector transfected cell. BRCA2-1335 is less effective. It can reduce the expression of BRCA2 by 45%. We planed to use these two RNAi construct to established stable cell lines of 76N. By isolating numerous clones, we should be able to obtain stable cell lines expressing varied level of BRCA2 comparing to the control parental cells. Cell lines with about half of the expression of level of the parental cells may be obtained. We will treat these cells with  $\gamma$ -radiation and asses their susceptibility of these cells to radiation induced transformation. Although these cell lines will not be same as the BRCA2 heterozygous cell lines, we should be able to assess the effect of reduced BRCA2 expression on the susceptibility of these cells to radiation induced transformation. The data obtained here should further corroborate the data obtained with the BRCA2 heterozygous cells.

## KEY RESEARCH ACCOMPLISHMENTS

- Constructed the positive-negative selection construct.
- Constructed the positive control construct.
- Standardized the PCR screen condition.
- Confirmed the status of BRCA2 in a normal mammary epithelial cell strain, 76N is normal and is suitable to be used to generate the BRCA2<sup>+/+</sup> cell line.
- Generated two promoterless constructs with puromycin and neomycin resistance gene respectively, which has been reported to have a higher success rate.
- Designed another set of knock out construct to target the exon 3 region of BRCA2.
- Developed two BRCA2 RNAis that can decrease the expression level of BRCA2.

## REPORTABLE OUTCOMES

An Abstract titled "susceptibility of brca2 heterozygous normal mammary epithelial cells to radiation induced transformation" was presented in the Era of Hope, which was held in Orlando, Florida on September 25-28, 2002.

## CONCLUSIONS

In the approved Statement of Work, we proposed three tasks for the first years: Task 1. Generate the BRCA2 Knock out construct and the positive control template constructs, sequence the construct. Task 2. Confirm the status of BRCA2 is normal in normal human mammary epithelial cells. Task 3. Transfect the knock out construct into normal mammary epithelial cells and screen for clones with one allele of BRCA2 disrupted. We have successfully accomplished Task 1 and 2. We already successfully constructed the positive-negative selection construct that was proposed in our grant application. We also constructed the positive control construct. Both constructs have been sequenced and proven to be correct constructs. We confirmed the status of BRCA2 in a normal mammary epithelial cell strain, 76N is normal and is suitable to be used to generate the BRCA2 <sup>+/+</sup> cell line. However, Task 3 turned out to be much more difficult than we have anticipated. Thus far we have not yet obtained clones with one allele of BRCA2 disrupted even after numerous attempts. Our experience of failure is in accord with the common notion that creating a knockout cell line is a time consuming, labor-intensive process with an uncertain success rate. However, to address an important problem such as the one proposed here, persistent effort is warranted. We are currently in the process of more attempts with or without modified strategy. We already generated two promoterless constructs with puromycin and neomycin resistance gene respectively, which has been reported to have a higher success rate. We already transfected both constructs into 76TERT cells and are in the process of selection. If we are able to obtain the BRCA2 <sup>+/+</sup> cells, we will perform the radiation transformation experiments as we proposed (Fig. 1). Otherwise, we will construct the set of knock out construct to target the exon 3 region of BRCA2 as shown in Fig. 5.

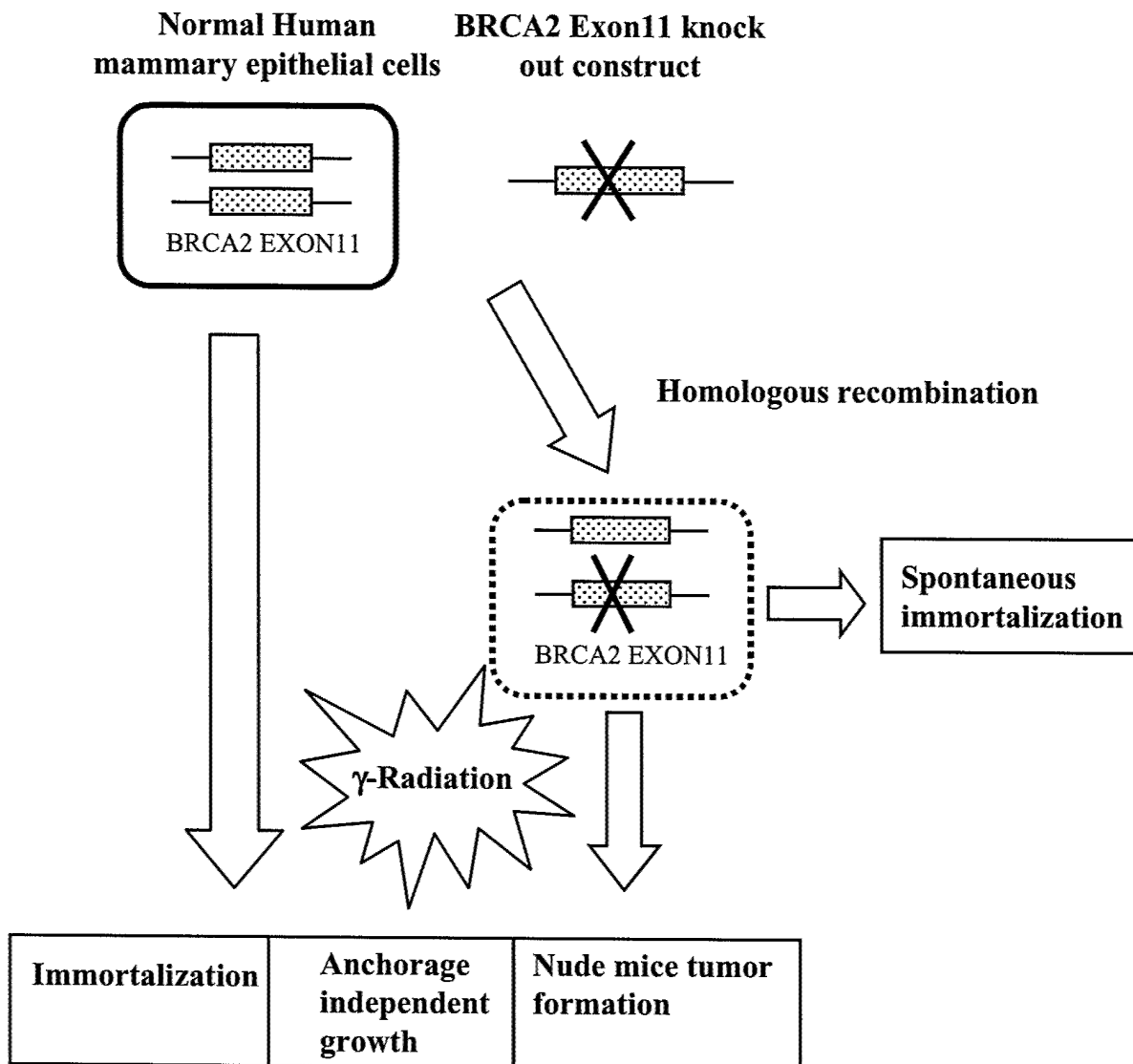


Fig. 1. Generation of a BRCA2 heterozygous mammary epithelial cell strain and comparing the susceptibility of the BRCA2<sup>+/-</sup> cells and its parental cells to radiation-induced transformation.

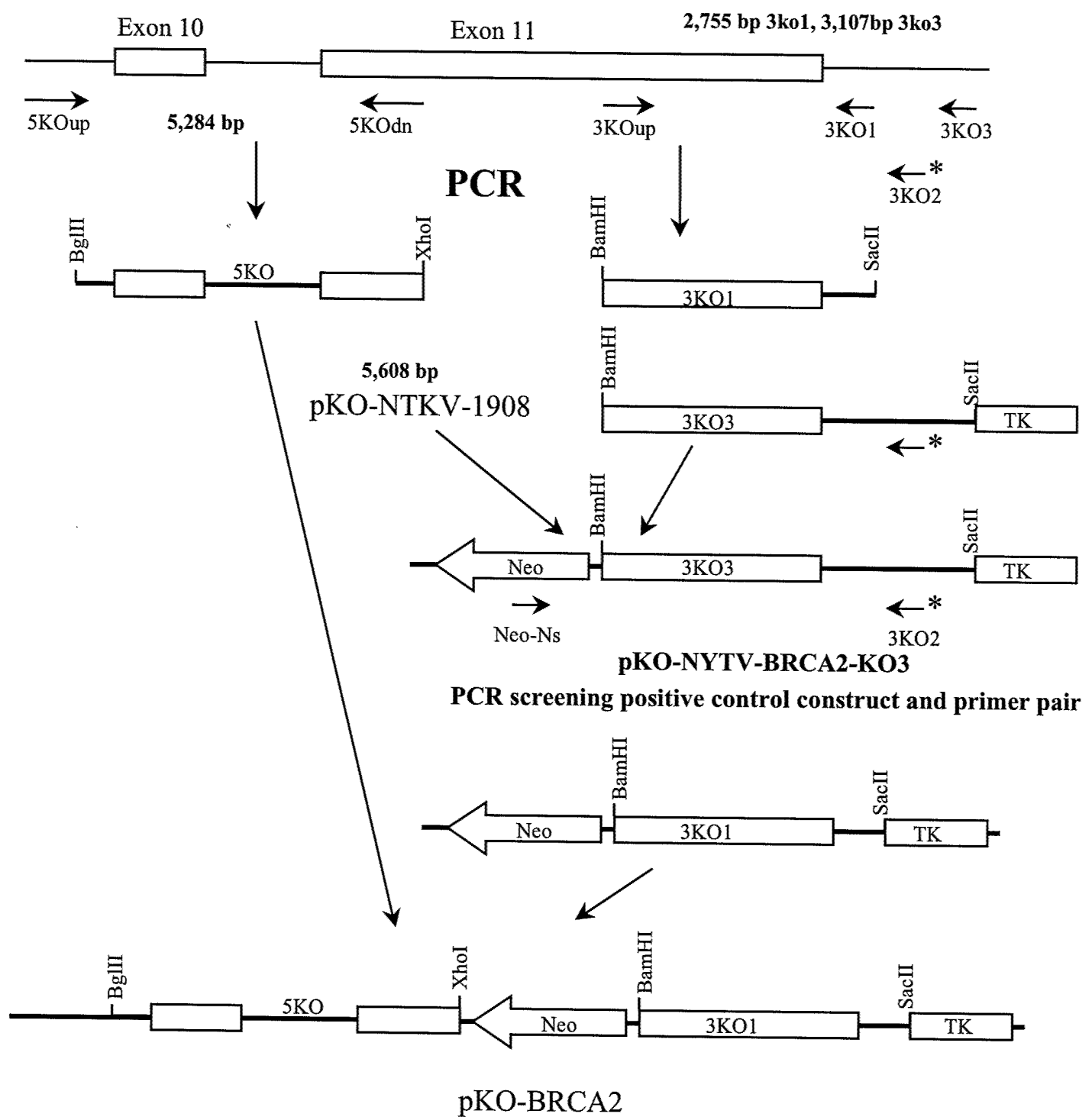


Fig. 2a. Schematic of the positive negative selection BRCA2 knockout vector and the positive control plasmid design.

# pKO Scrambler NTKV-1908

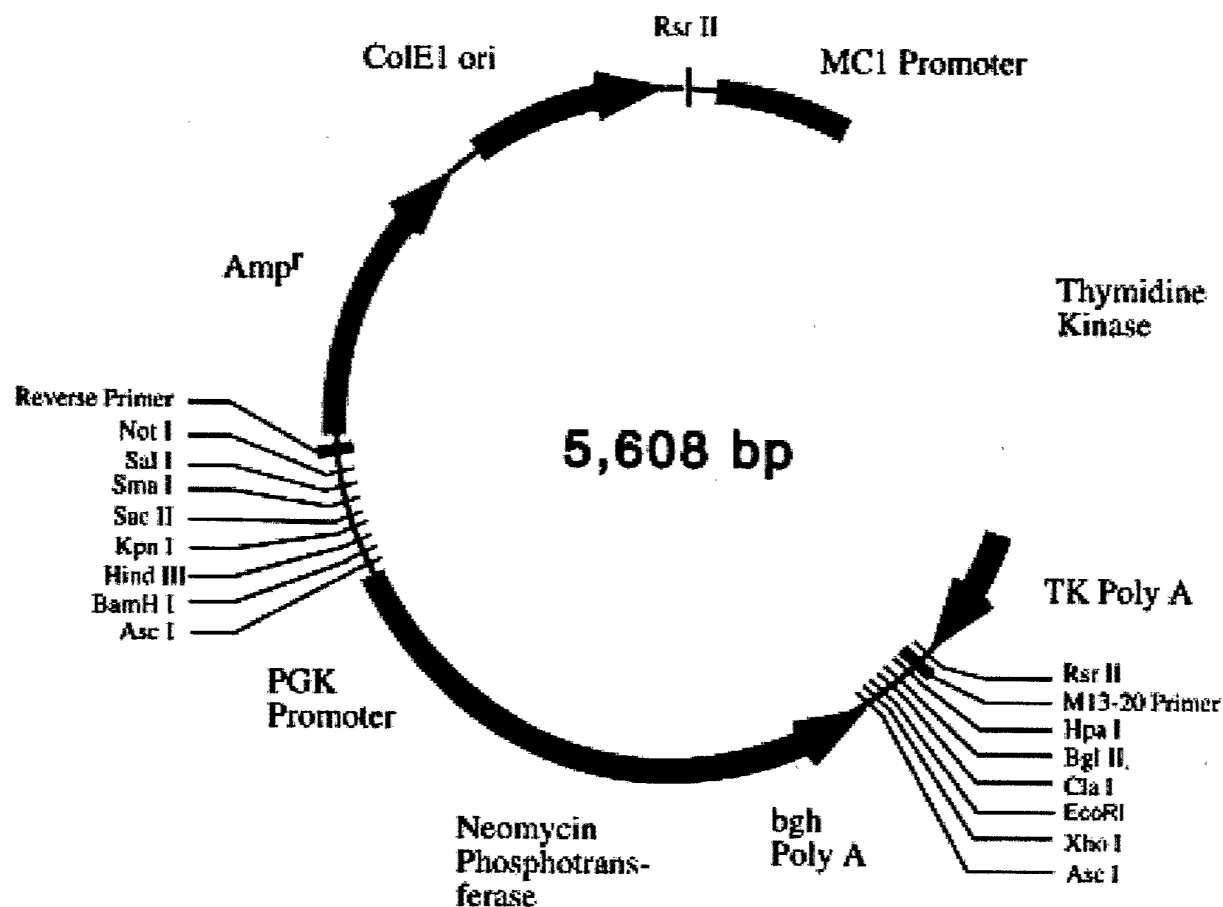


Fig. 2b. Schematic of pKO NTKV-1908.

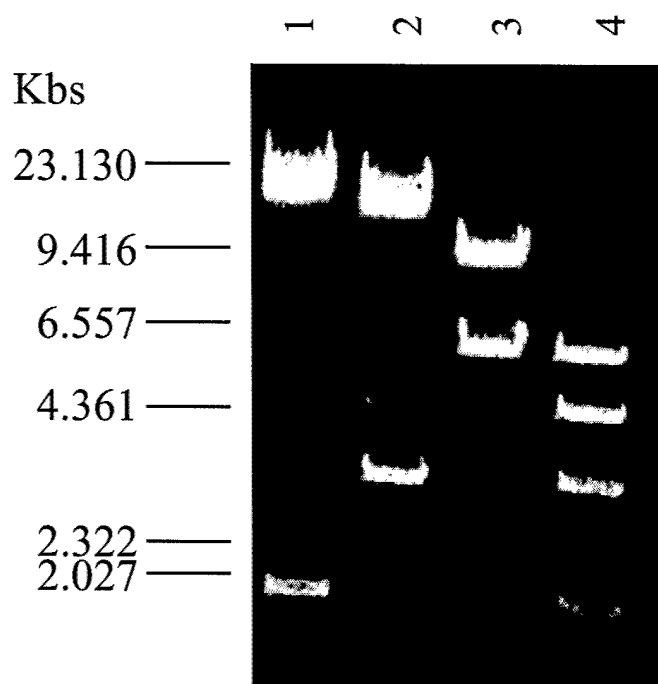


Fig. 2c. Restriction digestion analysis of pKO-BRCA2. Lane 1: pKO-NTKV-BRCA2 was digested with BamHI plus XhoI. Lane 2: digestion by XhoI plus BglII. Lane 3, digestion by SacII plus BamHI. Lane 4: digestion by XhoI, BglII, SacII and BamHI.



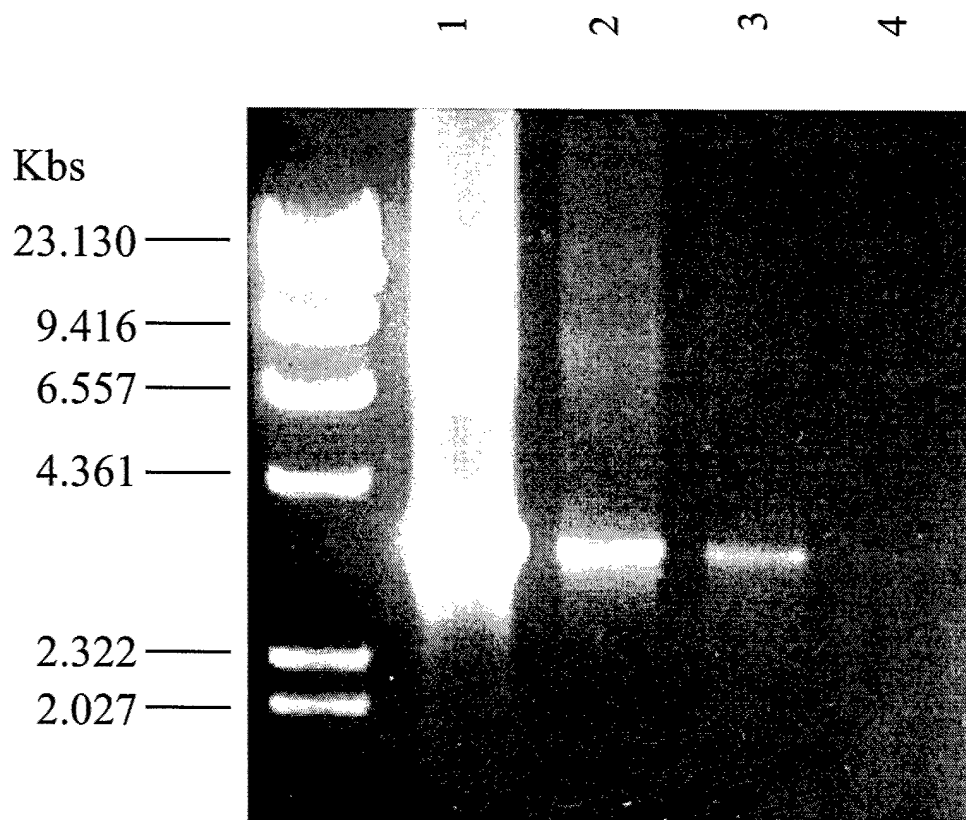


Fig. 3. PCR detection of the positive control plasmid. To standardize the PCR condition to screen for the correctly recombined clones,  $1.5 \times 10^{-7} \mu\text{g}$ ,  $1.5 \times 10^{-8} \mu\text{g}$ ,  $1.5 \times 10^{-9} \mu\text{g}$  or  $1.5 \times 10^{-10} \mu\text{g}$  of pKO-NYKV-BRCA2-3ko3 plasmid was mixed with  $2 \mu\text{g}$  of 76N genomic DNA to be used as the positive control template. The Expand Long Template PCR System was used to carry out the PCR using the following condition: initial denaturation  $94^\circ\text{C}$  2 min, denaturation  $94^\circ\text{C}$  30 seconds, Annealing  $65^\circ\text{C}$  30 seconds, elongation  $68^\circ\text{C}$  3 minutes, 10 cycles, then denaturation  $94^\circ\text{C}$  30 seconds, Annealing  $65^\circ\text{C}$  30 seconds, elongation  $68^\circ\text{C}$  3 minutes + 20 s for each successive cycle, 30 cycles. Lane 1:  $1.5 \times 10^{-7} \mu\text{g}$  (9000 copies), Lane 2:  $1.5 \times 10^{-8} \mu\text{g}$  (900 copies), Lane 3:  $1.5 \times 10^{-9} \mu\text{g}$  (90 copies), and Lane 4:  $1.5 \times 10^{-10} \mu\text{g}$  (9 copies).

# pKO-BRCA2

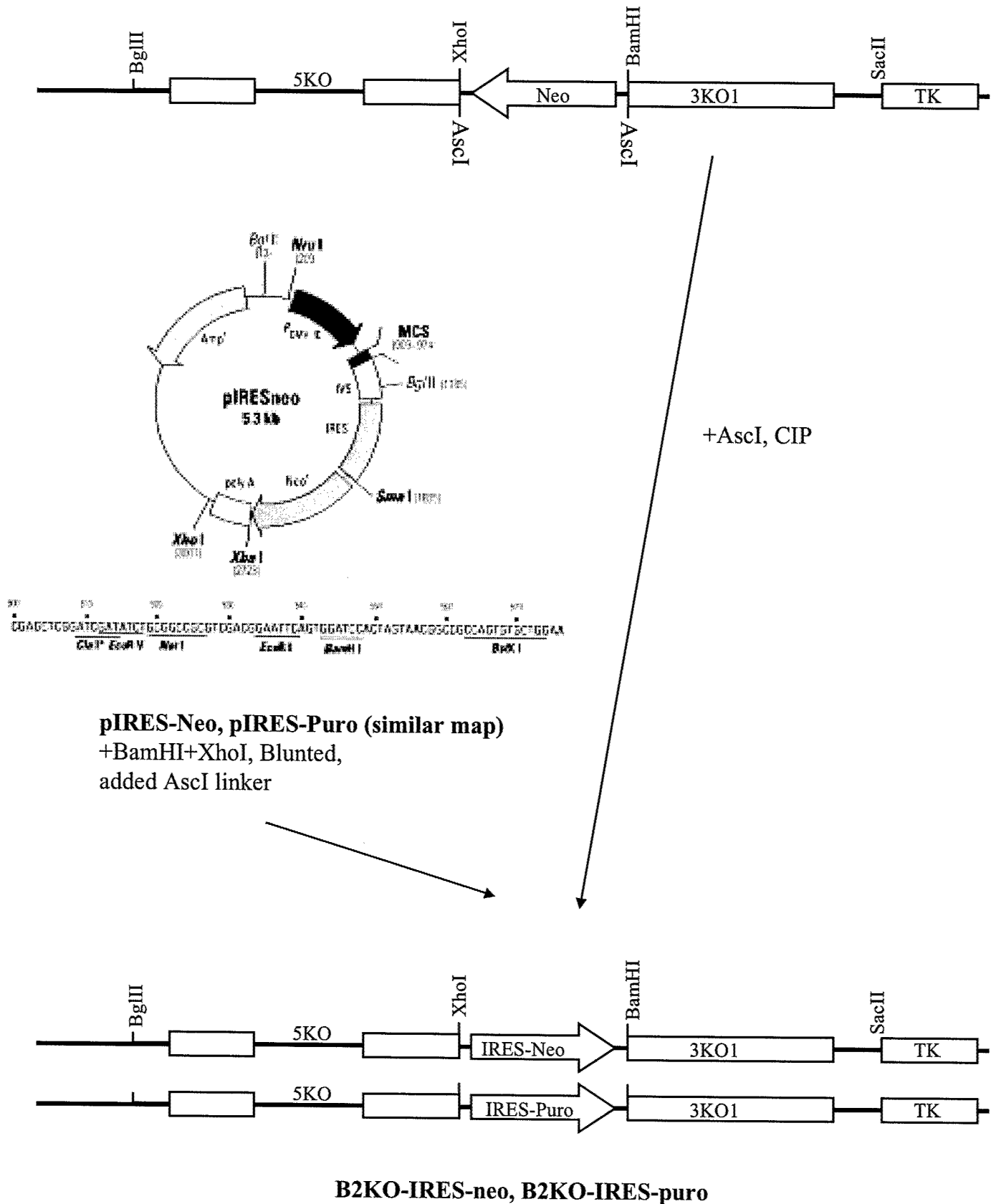


Fig. 4a. Schematic of the promoterless BRCA2 knockout design.

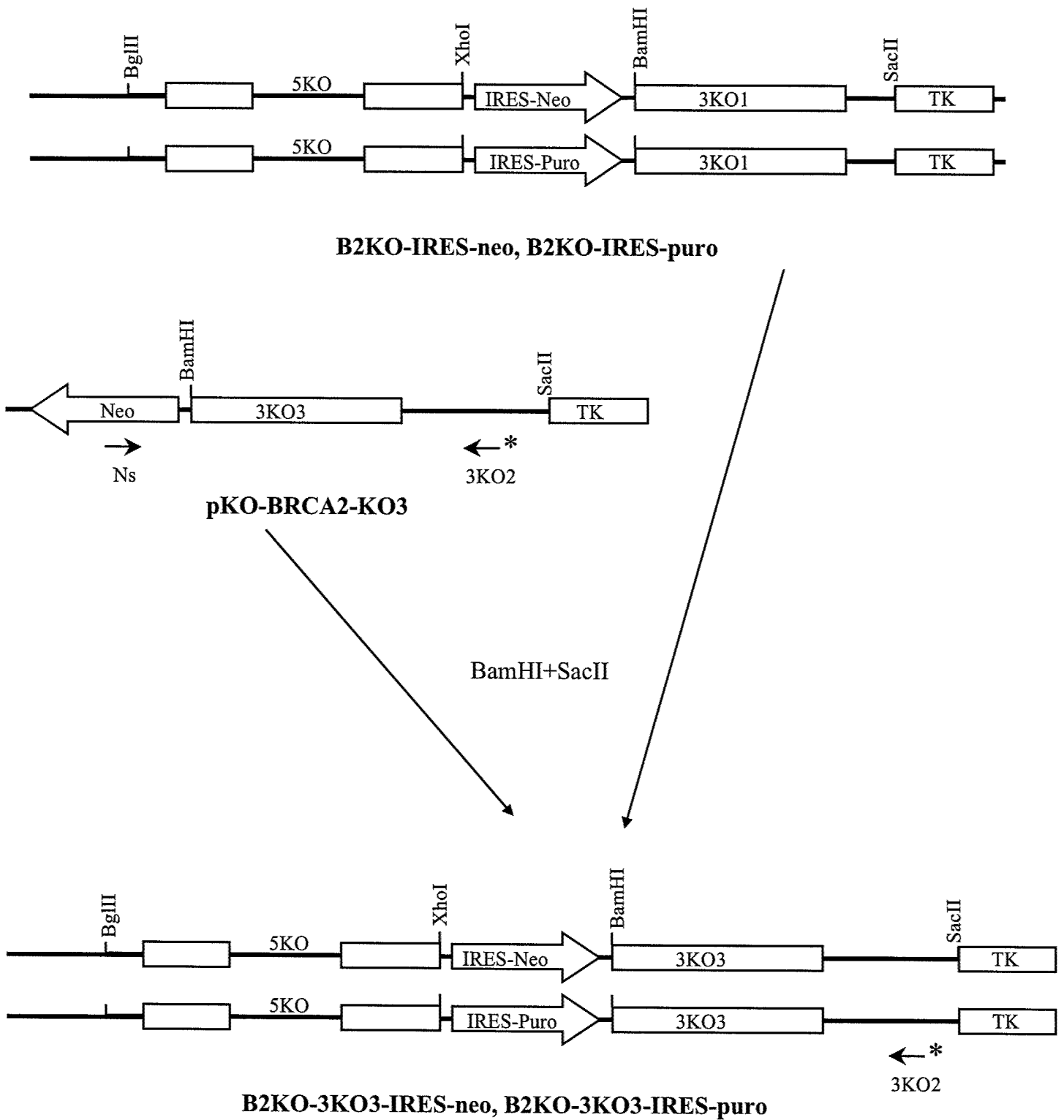


Fig. 4b. Schematic of the positive control plasmids design for the promoterless BRCA2 knockout vector.

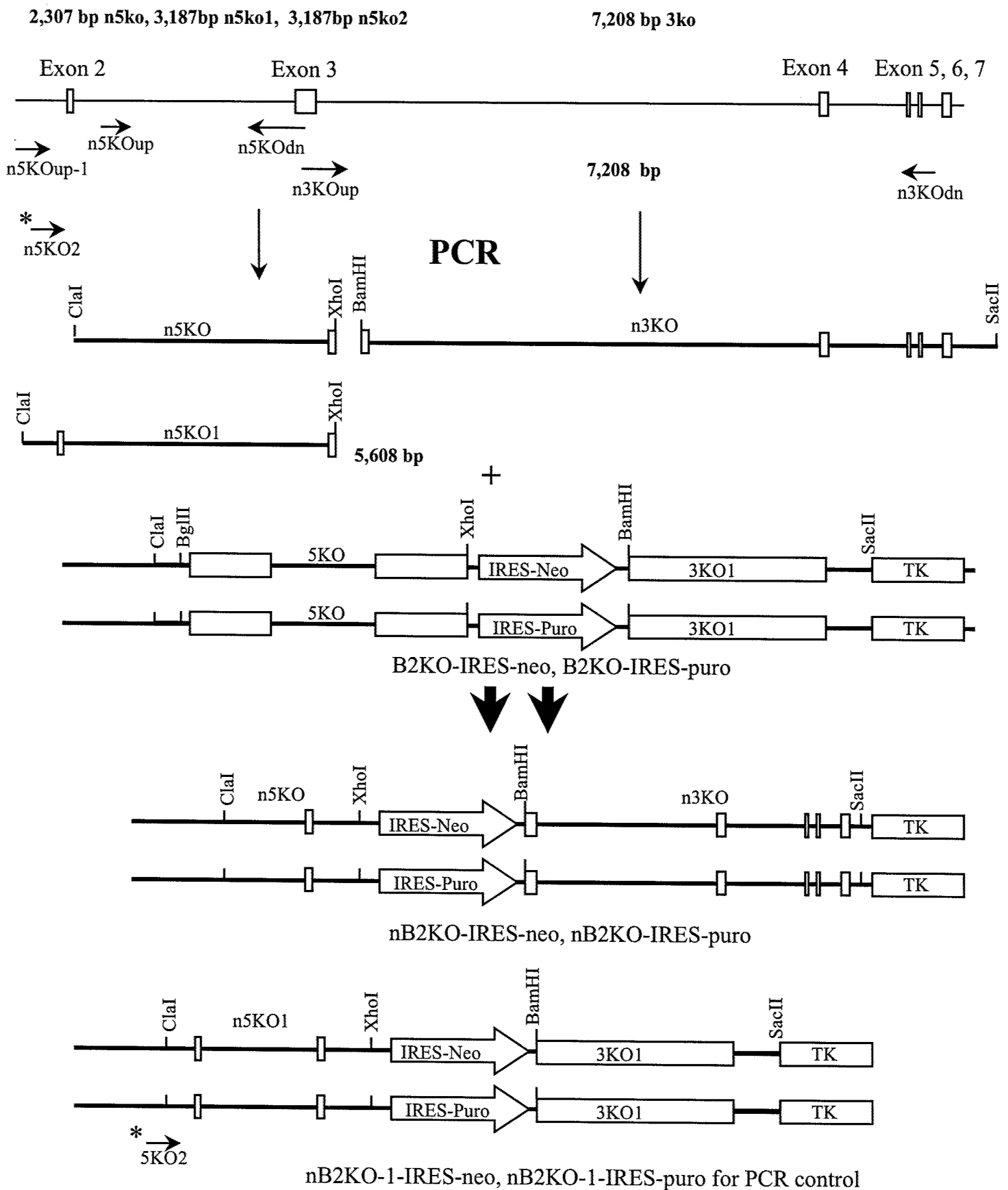


Fig. 5. Schematic of exon 3 promoterless BRCA2 knockout vector and its positive control plasmids design.

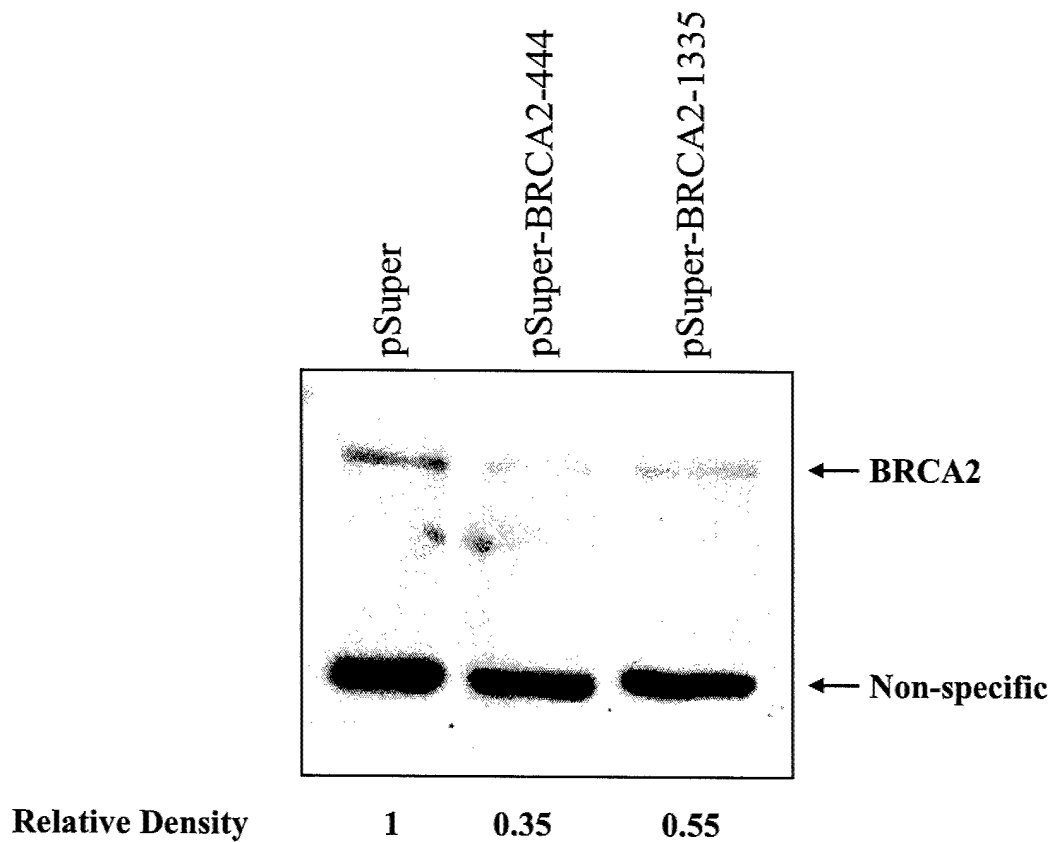


Fig. 6. BRCA2 western blotting analysis of 76Tert cells transfected with BRCA2 RNAi. 76Tert cells was transfected with pSuper-BRCA2-444 and pSuper-BRCA2-1335. The cells was harvested 72 hours after transfection in Sample Buffer and fractionated on a 5% SDS-PAGE gel. Western Blotting was performed using anti-BRCA2 antibody Ab1 (oncogene science). This antibody detects a non-specific band in these cells. The density of BRCA2 bands and the non-specific bands were quantified with NIH image and the relative density was shown with the density of BRCA2 band from pSuper vector transfected cells as one.

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